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# Neem-tree (*Azadirachta indica* Juss.) extract as a feed additive against the American dog tick (*Dermacentor variabilis*) in sheep (*Ovis aries*)

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ABSTRACT

Acaricides can be conveyed to ticks *via* the blood of their hosts. As fruit and kernel extracts from the *Meliaceae* family, and, in particular the tetranortriterpenoid azadirachtin (AZA) inhibits tick egg production and embryogenesis in the *Ixodidae* ticks, we investigated the effects of Neem Azal®, an extract containing 43% AZA, given as a feed additive to lambs artificially infested with engorging adult *Dermacentor vairiabilis* ticks. After tick attachment, the lambs were allotted to three dietary treatments: AZA0 (control, $n = 10$), AZA0.3 ($n = 5$), and AZA0.6 ($n = 5$), with feed containing 0%, 0.3%, and 0.6% AZA on DM basis, respectively. In half of the AZA0 lambs, ticks were sprayed on day 4 after attachment with an ethanol:water:soap emulsion containing 0.6% AZA (AZA0S). In spite of its very pungent odor, the neem extract was well accepted by all but one lamb. No differences were found between treatment groups in liver enzymes in blood, and there was no indication of toxicity. The plasma AZA concentrations after 7 and 14 days of feeding AZA were (4.81 and 4.35 μg/mL) for the AZA0.6 and (3.32 and 1.88 μg/mL) for the AZA0.3 treatments, respectively ($P < 0.0001$). Treatments were not lethal to ticks, but tick weights at detachment were 0.64, 0.56, 0.48, and 0.37 g for ticks from the AZA0, AZA0.3, AZA0S, and AZA0.6 treatments ($P < 0.04$), respectively, suggesting that blood AZA impaired blood-feeding. The highest mortality rate after detachment was for AZA0.6 ($P < 0.09$). As AZA affects embryo development and ticks at the molting stages, we expect that following treatments of hosts for longer periods, one-host ticks will be more affected than the three-host tick *D. variabilis*.


## 1. Introduction

As blood is the sole feed of engorging ticks, scientists have attempted to deliver acaricides systemically to ticks in tick-afflicted animals. For instance, the long-term delivery of the acaricide ivermectin (22, 23-dihydroavermectin $B_{1a}$ + 22, 23-dihydroavermectin $B_{1b}$) by a ruminal bolus was successful in controlling *Boophilus ainnulatus* (Taylor and Kenny, 1998; Miller et al., 2001), whereas sustained-release famphur boluses failed in keeping famphur blood levels high enough to control both lone star and Gulf Coast ticks feeding on Hereford heifers (Teel et al., 1979).

Natural acaricides are produced by a variety of plants: two South American species of *Stylosanthes* produce sticky secretions that immediately immobilize and kill larvae of *Boophilus microplus* (Sutherst et al., 1982). Cardiac glycosides produced by *Digitalis purpurea* L. (*Scrophulariaceae*) and *Calotropis procera* (Ait) R Br (*Asclepiadaceae*) are lethal to ticks (Al-Rajhy et al., 2003). Trees of the Meliaceae family, and in particular the neem tree (*Azadirachta indica*

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Juss.), synthesize a wide array of compounds, including a number of limonoids (tetranortriterpenoids) termed globally "azadirachtin" (AZA), which are lethal or impair development in a wide array of arthropods (Schmutterer, 1990). When eggs of *Hyalomma anatolicum excavatum* are immersed in neem extracts *in vitro*, hatching accelerates, and the mortality of newly hatched larvae increases (Abdel-Shafy and Zayed, 2002). Neem extract reduces feeding activity of *Hyalomma dromedari* larvae, prolongs the period for molting to nymphal stage, and reduces moltability by 60% (Al-Rajhy et al., 2003). Extracts of *Melia azedarach* inhibit egg production of immersed *B. microplus* ticks (Borges et al., 2003), and weekly spraying with neem-seed extracts decreases the number of ticks on goats (Schwalbach et al., 2003) and cattle (Webb and David, 2002) in Southern Africa.

Using AZA, a natural plant product, as a feed additive to combat ticks on livestock would be environmentally friendly. Provided that AZA is absorbed and not wholly detoxified by the liver, daily feeding would stabilize AZA concentrations in blood; as AZA is unstable in sunlight (Stokes and Redfern, 1982; Barrek et al., 2004), it would probably not be residual in the feces, in contrast with ivermectin (Iglesias et al., 2006).

A pre-requisite of using AZA against ticks would be its non-toxicity to hosts. Although AZA is toxic to murine hybridoma (Gotkepe and Plhak, 2003), an extract containing 12% AZA did not affect rats negatively when administered orally for 90 days at 1500 mg/kg/day (Raizada et al., 2001), and rats fed diets containing 50 mg/day/kg BW for three generations showed no impaired reproductive or growth performance (Srivastava and Raizada, 2007). However, we are not aware of reports on the toxicity of AZA given *per os* to ruminants and this issue needs investigation.

The objectives of the present study were to determine: (1) if AZA is toxic to a ruminant and (2) if AZA reaches measurable and efficacious blood concentrations against adult engorging ticks.

As a model, we used Neem Azal® containing 43% AZA as a feed additive. Lambs were infested with adult three-host American dog ticks *Dermacentor variabilis* L.

## 2. Materials and methods

### 2.1. Sheep management

The study was carried out in July–August 2008 at the Green Canyon Ecology Center, located at Utah State University in Logan, according to procedures approved by the Utah State University Institutional Animal Care and Use Committee. Twenty Suffolk crossbred lambs, with an average BW of 45.7 (s.d. 4.3) kg, were individually penned outdoors, under a protective roof in individual, adjacent pens measuring 2.4 × 3.6 m. They were provided freely with water and trace-mineral-salt blocks *ad libitum*. The pre-experimental daily ration (1 kg of Dry Matter, DM) consisting of 840 g of alfalfa pellets and 300 g of ground corn (as fresh), was fed in two meals at 0600 and 1600. After 10 days adaptation to the basic diet and the pens, wool covering the intersection regions of the ears and upper head, and a 20 cm × 20 cm area around the L5 (fifth lumbar vertebra), was shorn to the skin. Four 6-cm lengths of 8-cm diameter surgical cotton stockinettes were glued along each animal's two ears and midline at the fifth lumbar vertebra (L5) using a commercially available adhesive (Nasco Livestock ID Tag Cement, Ft. Atkinson, WI, USA).

### 2.2. Tick management

On July 1 (day 1, see Fig. 1), 400 adult *D. variabilis* ticks were purchased at *the Tick Rearing Facility* of *Oklahoma State University* (Stillwater, OK). Five ticks, including two or three males were introduced into each stockinette and open end was twisted into a pigtail and secured closed with rubber bands. Stockinettes were checked daily for the presence of males, as adult females of the three-host *D. variabilis* do not engorge fully unless fertilized. Lambs began feeding on the experimental diets on day 5, after tick attachment was verified. On that date, 205/400 ticks of both genders were counted on animals, with the losses a result of sheep tearing off stockinettes and chewing rubber bands. Attachment of the remaining ticks was 90.7%. In other words, approximately 90 attached female ticks were the initial populations used in the experiment.

Detachment of the ticks from the sheep began on day 10. Only 46 ticks were finally harvested and weighed until all ticks detached (day 17), as the others were crushed dead by self-grooming lambs.

The harvested ticks were kept individually at 23 °C in Petri boxes covered with bandage cotton cloth in a humidity chamber consisting of a covered tub with 5 cm of $K_2SO_4$ supersaturated salt solution (Winston and Bates, 1960). Oviposition occurred between day 20 and day 32. Tick mortality and individual egg production (by weight) were quantified for each tick.

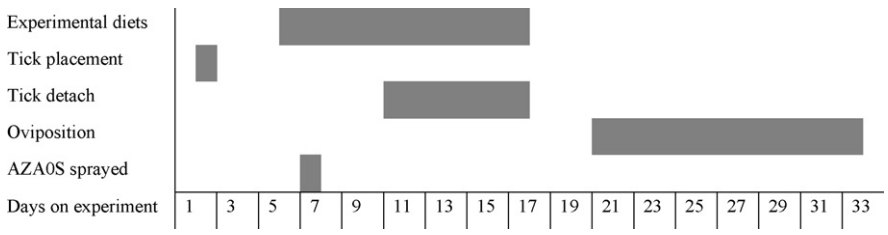


**Fig. 1.** Time schedule of experimental procedures.

**Table 1**
Experimental rations.

| | AZA0 | AZA0.3 | AZA0.6 |
|---|---|---|---|
| *On fresh matter basis* | | | |
| Corn grain (g/day) | 300 | 150 | 0 |
| Corn grain with 4.8% Neem Azal (g/day) | 0 | 150 | 300 |
| Alfalfa pellets (g/day) | 840 | 840 | 840 |
| Total feed (as fed) | 1140 | 1140 | 1140 |
| Neem Azal (g/day) | 0 | 7.2 | 14.4 |
| AZA (g/day) | 0 | 3.1 | 6.1 |
| *On dry matter basis* | | | |
| Total feed (on DM basis) | 1003 | 1003 | 1003 |
| AZA (% of DM) | 0 | 0.3 | 0.6 |

### 2.3. Experimental treatments

Lambs were allotted to three treatments with diets containing 0% AZA (AZA0, $n = 10$), 0.3% AZA (AZA0.3, $n = 5$) and 0.6% AZA (AZA0.6, $n = 5$) on DM basis. This was done by distributing 150 g/day (half of daily provision of ground corn grain) and 300 g/day (all of the daily provision of corn grain) in the form of 4.8% AZA premix to the AZA0.3 and AZA0.6 lambs, respectively (Table 1). Experimental diets were fed until day 17. The source of AZA was NeemAzal® Technical (E.I.D. Parry Ltd., BioProducts Division, Thyagavalli, India) containing 42.7% AZA. The quality of diet mixing was assessed by duplicate analysis of azadirachtin in samples collected five times between day 2 and day 17.

On day 7, two days after experimental feeding was initiated, the ticks of five lambs – termed the AZA0S treatment—from the AZA0 group were sprayed with an emulsion containing 0.6% AZA. The emulsion was prepared by dissolving 28.1 g of Neem Azal® in 600 mL of ethanol 96%, and adding tap water and a commercial soap to a volume of 2 L, with thorough mixing for 1 h.

### 2.4. Blood sampling

Sheep were bled by jugular venipuncture ($2 \times 10$ mL) on three occasions at 0800: before experimental feeding was initiated (day 1), when ticks started detaching (day 7) and after all ticks had detached (day 14). Blood was held at 4 °C, centrifuged, separated from the clot within 2 h, and frozen (−20 °C) until serum chemistry analysis.

Complete blood counts (CBC) were carried on whole blood collected in EDTA using a Coulter® LH 750 hematology analyzer (Beckman Coulter, Inc.; Fullerton, CA). Whole blood smears were dried and fixed within 2 h after collection. The complete blood count included white blood cell count (WBC), red blood cell count (RBC), red blood cell distribution width (RDW), packed cell volume (PCV), hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Standard serum chemistry included albumin, blood urea nitrogen, creatinine, and bilirubin. Liver enzymes (alkaline phosphatase, AP; Lactic dehydrogenase, LDH; alanine transaminase, ALT; and aspartase amino-transferase, AST) were also determined, using an Ortho Vitros 950 (Ortho-Clinical Diagnostics Inc., Markham, Ontario, Canada) with a patented "Microslide" and enhanced chemo-luminescence technology.

### 2.5. Azadirachtin analysis

Although AZA is routinely assessed by HPLC (Barrek et al., 2004), to our knowledge assays for blood concentrations of AZA have not been published.

#### 2.5.1. General

Acetonitrile was HPLC grade (Burdick and Jackson, Muskegon, MI). Water was filtered and deionized by Waters Pro PS (18 mΩ). Azadirachtin standard was technical grade (PS 2075, Supelco, Sigma-Aldrich, StLouis, MO). HPLC–MS system was Thermo Finnigan LCQ Advantage Max with APCI ionization source.

#### 2.5.2. Feed AZA

The AZA concentration of the Neem Azal® was first established in India after dissolving in acetonitrile:water (35:65) and assayed by HPLC with UV detection at 215 nm (E.I.D. Parry Ltd., BioProducts Division, Thyagavalli, India). This was done by using a Shimadzu Liquid Chromatograph System Model LC-10AD withUV/VIS Detector Model mSPD-10A and Spinchrom CFR with a Luna C-18(2) column, 5 μm particle size, flow rate 1.0 mL/min, retention time: AZA-A, 19–20 min, AZAb, 20–21 min samples (25 μL) were injected by using a Hamilton (USA) micro-syringe.

#### 2.5.3. Plasma sample extractions and preparation

Plasma samples (1.0 mL aliquot) were placed into 7 mL vials. Acetonitrile (1.0 mL) was added in 200 μL aliquots (5×). After each addition of acetonitrile the samples were mixed and after the final aliquot of acetonitrile the sample was centrifuged. A 1.0 mL aliquot of the upper clear solution was removed with a pipette to a HPLC auto-sample vial for LC/MS analysis.

Feed samples (2.5 g aliquot) were placed into 50 mL polymer tubes. Acetonitrile (50 mL) was added and the samples mixed by mechanical rotation for 16 h. Samples were centrifuged and a 10 μL aliquot was added to 0.990 mL of 50% acetonitrile in an auto-sample vial.

#### 2.5.4. Preparation of standards

A stock solution of Azadirachtin was prepared by dissolving 2.3 mg of standard in 2.3 mL of acetonitrile. A 1/10 dilution of stock was prepared by diluting 0.100 mL of stock with 0.900 mL of 50% acetonitrile (water). Standards used in feed analysis were prepared by diluting 40 μL of stock with 0.960 mL of 50% acetonitrile which was then serially diluted by 1/2 to prepare standards at 20, 10, 5, 2.5, 1.25 μg/mL. Standards used in plasma analysis were prepared by diluting 0.030 mL of 1/10 stock with 0.970 mL of 50% acetonitrile, which was then serially diluted by 1/3 (0.333 into 0.667 mL of 50% acetonitrile) to prepare standards at 3.00, 1.00, 0.333, 0.111, and 0.037 μg/mL.

#### 2.5.5. LC/MS analysis

Standards and samples were analyzed by LC/MS using a Betasil C18 column (100 mm × 2.1 mm; Thermo Finnigan).

Injection volume was 25 μL. Solvent was an isocratic flow of 35% acetonitrile in 20 mM ammonium acetate at a flow rate of 0.300 mL/min. The column flow was connected directly to an APCI ionization source of the mass spectrometer. The mass spectrometer was set to detect a mass range of 150–800 $m/z$ and azadirachtin was quantified from peak areas of the reconstructed ion chromatogram for 685 $m/z$ ($MH^+-2H_2O$).

### 2.6. Statistical analysis

Tick and egg weights were analyzed by two-way analyses of variance (GLM; SAS, 1989) using a model with AZA treatment (0%, 0.3%, and 0.6% AZA), tick location (ear or L5), and their interactions; sheep were nested within treatments. Plasma azadirachtin, and serum biochemical and hematological measurements were analyzed using a repeated measurement procedure with sheep within treatments as the error term. A chi-square analysis was done to assess if tick actual mortality rates after detachment deviated from expected rates.

## 3. Results

### 3.1. Hematology and toxicity

The feeding behavior of lambs fed AZA showed that they identified the presence of novel component in their food in the first days of experiment. For lambs fed AZA, consumption of feed was occurring in discrete feeding bouts, compared with the control lambs that rapidly and continuously ate their rations. However, in spite of the pungent odor of AZA concentrates, only one lamb from the AZA0.6 treatment had to be replaced by a lamb from the AZA0.3 treatment on day 2 after experimental diets were initiated. No food residuals were noted thereafter.

The complete blood cell counts, blood cell volume measurements and measurements associated with hemoglobin were not affected by treatments (Table 2); nor did serum albumin or blood urea nitrogen, as estimates of protein status, differ among groups. No decrease in PCV and hemoglobin was noted throughout the experiment, i.e., tick blood-feeding impact was too minor to be associated with anemia.

Overall, concentrations of enzymes in serum (AP, LDH, ALT, and AST; Table 3) did not differ among treatments. In particular, there was no increase of liver enzymes following AZA feeding in the AZA0.3 and the AZA0.6 groups (Fig. 2), i.e., no indication of liver damage by AZA; lambs in the AZA0S had lower creatinine concentrations than AZA0 (Table 3), but no differences were found among lambs fed AZA at different levels. Also, they AZA0S lambs exhibited lower AP, AST, and LDH initial values (Fig. 2), which were taken into account by the repeated measurements analyses.

### 3.2. Feed and plasma azadirachtin

The concentration of AZA in the NeemAzal®-Corn premix (planned to be 4.8%) was 4.75, 4.53, 5.20, 4.08, and 5.54, in randomly collected samples, i.e., 4.8%, on average. In other words, lambs received the amount of AZA planned initially.

Over the whole experiment, plasma azadirachtin was highest for AZA0.6 (3.05 ± 0.22 μg/mL), followed by AZA0.3

**Table 2**
Concentrations of white blood cells (WBC, millions/mL), red blood cells (RBC, millions/mL), red blood cell distribution width (RDW, %), packed cell volume (PCV, %), hemoglobin concentration (Hbg, g/100 mL), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), and mean corpuscular hemoglobin concentration (MCHC, g/100 mL) in lambs subjected to different azadirachtin treatments (means ± SE).

| | AZA0 | AZA0.3 | AZA0.6 | AZA0S | $P<$ |
|---|---|---|---|---|---|
| WBC | 14.4 ± 5.4 | 13.6 ± 6.0 | 18.6 ± 6.7 | 15.3 ± 6.3 | 0.95 |
| RBC | 4.28 ± 0.27 | 4.58 ± 0.29 | 4.95 ± 0.33 | 4.34 ± 0.31 | 0.44 |
| PCV | 21.3 ± 1.43 | 22.3 ± 1.59 | 24.8 ± 1.78 | 21.8 ± 1.68 | 0.48 |
| Hbg | 11.4 ± 0.49 | 11.6 ± 0.55 | 12.7 ± 0.60 | 11.6 ± 0.58 | 0.40 |
| MCV | 49.7 ± 0.24 | 50.0 ± 0.27 | 50.2 ± 0.30 | 50.1 ± 0.28 | 0.63 |
| MCH | 27.0 ± 0.80 | 26.6 ± 0.89 | 25.7 ± 1.0 | 26.9 ± 0.94 | 0.77 |
| MCHC | 54.4 ± 1.75 | 53.3 ± 1.95 | 51.4 ± 2.17 | 53.8 ± 2.05 | 0.74 |
| RDW | 27.9 ± 0.78 | 28.1 ± 0.87 | 29.1 ± 0.97 | 27.4 ± 0.91 | 0.62 |

**Table 3**
Concentrations of blood urea nitrogen (BUN, mg/100 mL), creatinine (mg/100 mL), albumin (g/100 mL), bilirubin (mg/100 mL), alkaline phosphatase (ALP, U/L), lactic dehydrogenase (LDH, U/L), alanine transaminase (ALT, U/L), and aspartate transaminase (AST, U/L) in lambs subjected to different azadirachtin treatments (means ± SE).

| | AZA0 | AZA0.3 | AZA0.6 | AZA0S | $P<$ |
|---|---|---|---|---|---|
| BUN | 17.2 ± 0.69 | 16.9 ± 0.71 | 15.1 ± 0.86 | 15.5 ± 0.80 | 0.17 |
| Creatinine | 0.81 ± 0.02b | 0.76 ± 0.02ab | 0.75 ± 0.02ab | 0.71 ± 0.02a | 0.03 |
| Albumin | 2.80 ± 0.078 | 2.70 ± 0.080 | 2.86 ± 0.097 | 2.65 ± 0.092 | 0.37 |
| Bilirubin | 0.297 ± 0.050 | 0.381 ± 0.052 | 0.380 ± 0.063 | 0.333 ± 0.059 | 0.64 |
| ALP | 132 ± 16 | 145 ± 17 | 135 ± 20 | 124 ± 18 | 0.85 |
| LDH | 1284 ± 65 | 1333 ± 67 | 1396 ± 80 | 1126 ± 76 | 0.12 |
| ALT | 25.8 ± 1.3 | 25.3 ± 1.3 | 25.5 ± 1.6 | 26.2 ± 1.5 | 0.93 |
| AST | 93.6 ± 4.76 | 100.6 ± 4.94 | 96.7 ± 5.93 | 82.7 ± 5.61 | 0.15 |

The right column of this table provides a value of significance for all measurements.

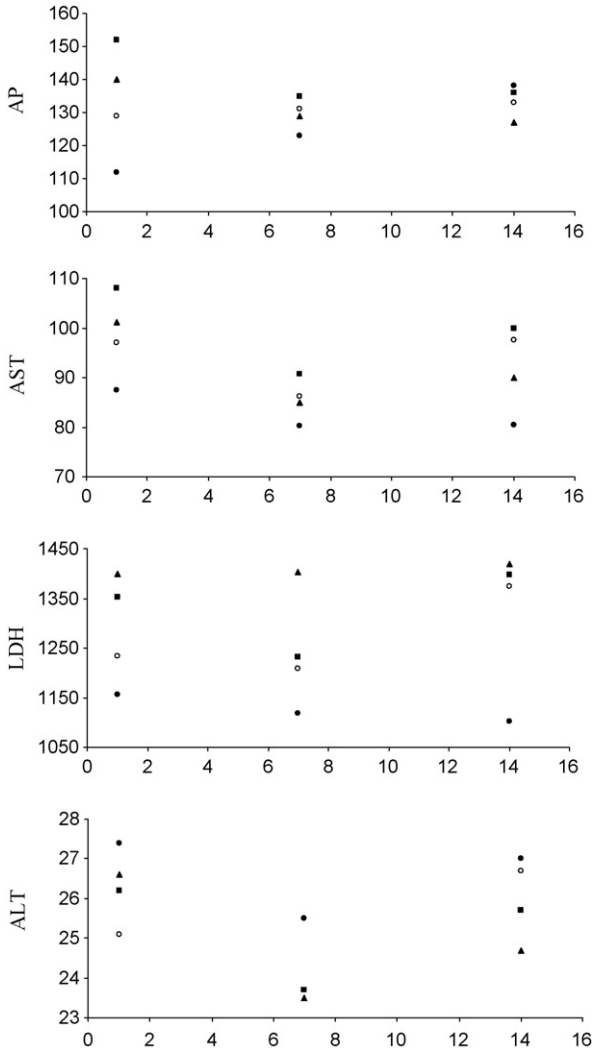


**Fig. 2.** Liver enzymes (alkaline phosphatase, AP; lactic dehydrogenase, LDH; alanine transaminase, ALT; and aspartase amino-transferase, AST) concentrations in the serum of lambs fed diets containing azadirachtin (AZA) at 0 (AZA0, ○), 0.3 (AZA0.3, ■), or 0.6% (AZA0.6, ▲) on dry matter basis from day 4. Lambs in the AZA0S (●) were sprayed with 0.6% AZA on day 7. *X*-axis: days on experiment and *Y*-axis: U/L.

($1.73 \pm 0.21$ μg/mL), and the AZA0 treatments (0 μg/mL). Treatments ranked AZA0.6 > AZA0.3 > AZA0 = AZA0S and all differences were significant at $P < 0.0001$. The plasma AZA concentrations after 7 and 14 days of feeding AZA were (4.81 and 4.35 μg/mL) for the AZA0.6 and (3.32 and 1.88 μg/mL) for the AZA0.3 treatments, respectively ($P < 0.0001$; Fig. 3). In other words, blood AZA was responsive to dietary AZA; and in AZA0.3, but not AZA0.6, the concentration of azadirachtin in plasma decreased ($P = 0.002$) from day 7 to day 14 of feeding AZA.

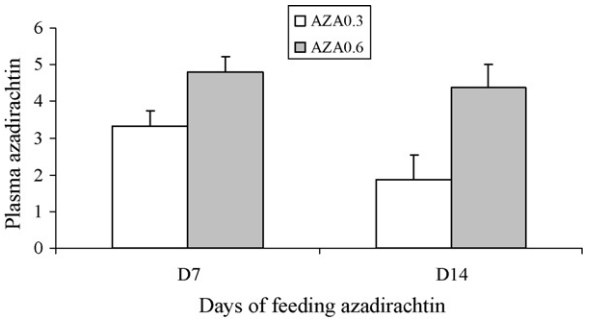


**Fig. 3.** Azadirachtin concentrations (μg/mL) in the plasma of lambs fed diets containing 0.3% (AZA0.3) or 0.6% (AZA0.6) on days 7 (D7) and 14 (D14) after feeding of azadirachtin was implemented: means ± SE. *X*-axis: days on experiment and *Y*-axis: azadirachtin (μg/mL).

### 3.3. Ticks

The task of maintaining functioning stockinette bags was difficult. Therefore, tick mortality until detachment cannot be directly associated with treatments, and is not analyzed here. We present results from 16 lambs, with $n = 5$, 4, 3, and 4 for the AZA0.6, AZA0.3, AZA0, and AZA0S groups, respectively, bearing 46 retrieved live female ticks, out of a possible 90 (Table 4).

When averaged among sheep, ticks attached near the ears weighed less than those attached at L5 (0.421 *vs.* 0.591 g, respectively, $P < 0.01$), but there was no interaction of attachment location and AZA treatment on blood-feeding. Tick weights were $0.367 \pm 0.054$, $0.562 \pm 0.047$, $0.642 \pm 0.067$, and $0.477 \pm 0.053$ g for the AZA0.6, AZA0.3, AZA0, and AZA0S, respectively (Table 4); ticks feeding on the AZA0 and AZA0.3 lambs weighed more ($P < 0.04$) than ticks on the AZA0.6 lambs, even though AZA0.6 ticks detached later. Tick weights in the AZA0S and AZA0.3 treatments did not differ significantly (Table 4).

Four out of 46 detached female ticks died before oviposition, of which 3 of 11 ticks were from the AZA0.6 group, and 1 of 13 was from the AZA0S group. These were the three smallest and second smallest ticks of their groups, respectively. The distribution of mortality deviated from expected at $P = 0.09$ (chi-square). The average egg weight was $89 \pm 3.9$ μg. The mean weight of eggs ($0.242 \pm 0.009$ g), as averaged per sheep, was not affected by treatments (Table 4).

**Table 4**
The effect of tick placement location (loc; ear or L5) and azadirachtin treatments on tick attributes (least square means within lambs ± SE).

| | AZA0 | AZA0.3 | AZA0.6 | AZA0S | Effects ($P<$) | | |
|---|---|---|---|---|---|---|---|
| | $N = 3$ | $N = 4$ | $N = 5$ | $N = 4$ | AZA | Loc. | AZA × loc |
| Tick weight at detachment[a] | 0.642 ± 0.067a | 0.562 ± 0.047a | 0.367 ± 0.054b | 0.477 ± 0.053ab | 0.04 | 0.005 | 0.68 |
| Days to detachment[a] | 10.2 ± 0.36ab | 9.5 ± 0.24b | 10.6 ± 0.27a | 9.1 ± 0.28b | 0.006 | 0.13 | 0.96 |
| Egg weight | 0.281 ± 0.04 | 0.267 ± 0.025 | 0.203 ± 0.033 | 0.229 ± 0.030 | 0.22 | 0.11 | 0.84 |

[a] Row-wise, least square means with different letters differ.

## 4. Discussion

Our objectives were to determine if AZA is toxic to ruminants and if AZA reaches peripheral blood, and if so, is it in concentrations needed to affect adult, engorging ticks. We found no notable toxic effect, i.e., no liver or kidney damage, as estimated from serum enzymes (Table 3; Fig. 2), for lambs fed AZA at a maximal concentration of 131 mg/kg BW/day for 2 weeks. This is in contrast with the deleterious effect of AZA on murine hybridoma (Gotkepe and Plhak, 2003), but in agreement with findings of no toxic effect of AZA administered orally for 90 days at 1500 mg/kg BW/day in rats (Raizada et al., 2001). If expressed on metabolic body weight, the AZA dose used by Raizada et al. (2001) is more than twenty-fold higher than in our study. In a long-term study, rats fed diets containing 50 mg/day/kg BW for three generations showed no impaired reproductive or growth performance (Srivastava and Raizada, 2007). In other words, it is improbable that AZA at doses used in the present study represents a toxicity hazard when given *per os* for tick control in sheep, and dietary AZA can probably be increased safely from levels used in this study.

The concentration of AZA in blood plasma was dose-dependent (Fig. 3). This is the first evidence, to our knowledge, that AZA given *per os* reaches, at least partly, the peripheral circulation of mammals without chemical modification. The decrease of AZA concentration in the plasma of lambs in the AZA0.3, but not the AZA0.6 group, suggests that AZA detoxification or biochemical modification might be enhanced after 1 week of low level administration, and this possibility warrants further investigation.

The absorption of AZA in mammals has not been quantified, to our knowledge. However, the bio-availability of limonin, a citrus limonoid, has been studied in humans (Manners et al., 2003). Following doses of 2 g (33 mg/kg BW) of limonin glycoside, a maximum of 5.27 nmol/L of limonin (approximately 3.0 μg/L) was found in the plasma. By comparison, the AZA concentrations in sheep plasma in our study were $10^3$ higher. This suggests that AZA is relatively resilient to ruminal degradation and liver detoxification, which qualifies AZA as candidate for systemic anti-tick control agent.

Stockinettes were placed at the base of ear because we assumed that blood availability would be high at this natural site of attachment and because *D. variabilis* favors head sites in humans (Felz and Durden, 1999). However, the L5 site was superior, and the site factor had to be added to the statistical analysis. We are not aware of other reports of tick engorging performance when placed at different sites on animals, but the L5 will be preferred in further experiments. All blood measurements, and in particular RBC, PCV, and hemoglobin clearly show that the number of ticks attached did not represent a challenge for sheep. By gluing all stockinette bags at the L5 site and stocking more ticks in each bag, a more meaningful tick challenge can be achieved.

Spraying an emulsion of 0.6% AZA was not immediately lethal to ticks, but the low percentage of tick recovery limits our conclusions. AZA given *per os* was not lethal to ticks, but tick weights at detachment were 0.64, 0.56, and 0.37 g for ticks from the AZA0, AZA0.3, and AZA0.6 treatments ($P < 0.04$; Fig. 2), suggesting that blood AZA impaired blood-feeding in ticks. In addition, the higher ($P = 0.09$) mortality of AZA0.6 ticks after detachment is consistent with findings *in vitro*. Abdel-Shafy and Zayed (2002) reported dose-dependent mortality rates with unfed *H. anatolicum excavatum* adults immersed in AZA solutions: at 0.6% of AZA, 68% of mortality was recorded on the first day of treatment, accumulating to 100% after 2 weeks. Borges et al. (2003) reported a dose-dependent reduction in the egg production rate of engorged ticks immersed in solutions of a *M. Azedarach* hexanic extract, with total suppression of oviposition by immersion in a solution of 0.25% extract.

In contrast with all other studies of the effect of AZA on ticks, the present study was conducted *in vivo*, and ticks were constantly exposed to the acaricide in blood. Blood AZA concentration of approximately 4.6 μg/mL, but not concentrations ranging from 1.9 to 3.2 μg/mL (Fig. 2), seemed to impair engorging, suggesting a threshold above 3.2 μg/mL. The lowest AZA concentration used by Abdel-Shafy and Zayed (2002) in their immersion study *in vitro* was 1.6% of Neem Azal (5% AZA), i.e., 800 μg/mL, which is 138-fold the AZA whole blood (corrected for cell volume) concentration in our study. The 800 μg/mL dose was associated with 30% mortality on the first day, accumulating to 92% after 2 weeks. Al-Rajhy et al. (2003) reported $LC_{50}$ values of 5000 μg/mL for *H. dromedarii* ticks exposed to AZA *in vitro*. Our data suggest that chronic exposition could harm ticks at much lower doses of AZA *in vivo* than those used for *in vitro* studies. As ivermectin ruminal boluses are successfully used for *B. annulatus* control (Taylor and Kenny, 1998), there is no doubt that blood can be an adequate medium to convey acaricides into ticks. However, the level of tick control reached by using these boluses is dose-dependent (Miller et al., 2001). Therefore we assume that a higher level of dietary AZA than used in the present study is needed to reach an AZA blood concentration high enough to kill female adult ticks before or after detachment.

The major effect of AZA on arthropods is a disruption of development and, in particular, of the transformations from larvae to nymphs, and from nymphs to adults (Schmutterer, 1990). Indeed, the most dramatic effects of AZA on ticks *in vitro* were observed on developmental stages (Abdel-Shafy and Zayed, 2002; Al-Rajhy et al., 2003; Borges et al., 2003). Therefore, the choice of adult *D. variabilis* females to test the efficacy of AZA against ticks was not optimal, because of the very short lifespan and exposure to dietary AZA by ticks feeding on their 3rd host. One-host ticks, which spend a greater percentage of their life on one host, either as pre-adults or adults, would apparently be exposed to AZA for a longer period, and therefore would be more vulnerable. As AZA affects embryo development and ticks during molting, we expect that one-host ticks will be more affected than the three-host tick *D. variabilis*. This hypothesis deserves further investigation.

Finally, in addition to their acaricide properties, AZA (Bray et al., 1990) has anti-malarial properties. Similar limonoids, trichirubins have been found in leaves of *Trichilia rubescens* putatively ingested by chimpanzees as self-medication against malaria (Krief et al., 2004). This

raises the exciting possibility that ruminants might learn to ingest AZA when tick-challenged. As self-medication against ticks is not known, this opens a wide field of far-reaching investigations.

## 5. Conclusions

AZA from NeemAzal® Technical is not toxic to lambs when given *per os* for 12 days at 0.6% of diet on DM basis. Administration of AZA as feed supplement results in dose-responsive blood concentration of AZA. Inclusion of 0.6% of AZA in lamb feed seems to decrease blood-feeding in *D. variabilis* adult, female ticks, but further investigation with a larger number of lambs and ticks is needed to reach a final conclusion.

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